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March 7, 1974

Ms. Beverly J. Hungerford
Grant Administrator
National Aeronautics and Space Administration
Washington, D. C. 20456

Re: DHC-2 (B. J. Hungerford)
NASA No. NGR 26-003-~~013~~ completed 9-30-69
University of Missouri Research Fund Code No. 5814-2229

Dear Ms. Hungerford:

Your letter of January 31, 1974 to Mr. Rudy L. Koch, University of Missouri, Mngr., Research and Restricted Accounting, 118 University Hall, Columbia, Missouri 65201 has been referred to me.

A 15 page, Final Composite Progress Report has been already forwarded to Dr. Joe Saunders, NASA, on November 1, 1969. Enclosed is a copy for your records. If you wish additional copies, there are 20 or so copies on file in Dr. Joe Saunders' office, or you might duplicate this one if needed.

Page 14 of this report has a list of 10 publications from this NASA-supported research project. Since that was prepared in 1969, it is out of date. Therefore, I now enclose a revised bibliography that should be filed with the original report to Dr. Joe Saunders. Dr. Saunders has the reprints referred to therein (sent on May 5, 1970 and November 5, 1971).

I hope this Report meets your needs. If not, please advise me. We were certainly appreciative of the support for NASA for the two-year period during which this research grant was effective. If NASA sees a way in the future to continue this fundamental biochemical research which has also carry-over in bioregeneration-recycling, I hope you will so advise us. We have the laboratory expertise, procedural know-how, the intellectual resources and the literature knowledge to be able to continue this or closely related work.

Sincerely,

Robert L. Wixom, Ph.D.
Professor
Principal Investigator of earlier NASA
Research Grant NGR-26-003-23

cc: Dr. Joseph F. Saunders
Mr. Rudy L. Koch
Mr. Willis E. Linneman
Dr. Benedict J. Campbell
Dr. Robert H. Schiffman

(NASA-CR-138030) APPLICATION OF NITROGEN
METABOLISM IN AUTOTROPHIC BACTERIA TO
CHEMOSYNTHETIC BIOGENERATION IN SPACE
MISSIONS, SUPPLEMENT (Missouri Univ.)
18 p HC

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FINAL SUMMARY OF PUBLICATIONS
A SUPPLEMENT TO EARLIER FINAL COMPOSITE PROGRESS REPORT

Project Title: Application of Nitrogen Metabolism in Autotrophic Bacteria
to Chemosynthetic Bioregeneration in Space Missions.

Project Sponsor: National Aeronautics and Space Administration

Project Number: NASA No. NGR 26-003-²23; University of Missouri, No.
5814-2229

Project Initiated: 10-1-67 (fiscal start)

Principal Investigator: Dr. Robert L. Wixom
Associate Professor of Biochemistry and
Research Associate, Space Sciences Research Center,
School of Medicine
University of Missouri
Columbia, Missouri 65201

Period covered by earlier Final Composite Progress Report: 10-1-67 to 9-30-69;
prepared on 10-20-69.

Date of this supplement prepared: 3-5-74

Scope of this summary: This Summary includes all the publications from the
research supported by NASA and the Space Science Research
Center, University of Missouri. Reprints of all except
the last one have been previously forwarded to NASA.

List of Recent Publications Related to Grant

1. Wixom, R. L., Joseph, A. A., Hwang, S. W., and Semeraro, R. J., Comparative Aspects of Valine-Isoleucine Biosynthesis. Seventh International Congress of Biochemistry, Tokyo, Japan, August 19-25, 1967; Abstract.
2. Semeraro, R. J., and Wixom, R. L., The Pathway of Valine-Isoleucine Biosynthesis in Photosynthetic Bacteria. Trans. Mo. Acad. Sci. 1: 82 (1967); Abstract.
3. Joseph, A. A., and Wixom, R. L., The Biosynthesis of Valine and Isoleucine in Autotrophic Bacteria. Society for Experimental Biology and Medicine, Annual Meeting of the Missouri Section at the University of Missouri, Columbia, Missouri, December 9, 1967; Abstract.
4. Joseph, A. A., Amino Acid Metabolism in Propionibacteria and Hydrogenomonas. Thesis for Ph.D. degree, University of Missouri, December 19, 1968.
5. Wixom, R. L., and Joseph, A. A., Ammonia Assimilation in Hydrogenomonas. Eleventh West Central States Biochemistry Conference, November 2, 1968, Ames, Iowa; Abstract.
6. Wixom, R. L., and Semeraro, R. J., Valine Biosynthesis in Rhodopseudomonas spheroides. Federation Proc., 28: 910 (1969); Abstract.
7. Wixom, R. L., Joseph, A. A., and Becker, R. S. Amino Acid Metabolism in Hydrogenomonas eutropha. Abstract. 158th National Mtg., American Chemical Society, New York, New York, September 7-12, 1969.
8. Semeraro, Russell J., Valine-Isoleucine Biosynthetic Enzymes in Photosynthetic Bacteria, Ph.D. thesis, 1970, University of Missouri.
9. Joseph, A. A., and Wixom, R. L. Ammonia incorporation in Hydrogenomonas eutropha. Biochim. Biophys. Acta, 201: 295-299 (1970).
10. Wixom, R. L., Heinemann, M., Semeraro, R. J., and Joseph, A. A. Studies in valine biosynthesis. IX. The enzymes in photosynthetic and autotrophic bacteria. Biochim. Biophys. Acta, 244, 532-546 (1971).
11. Wixom, R. L. and Sheng, D. Y.-B. Utilization of amino acids and related nitrogenous compounds by Hydrogenomonas eutropha. Federation Proc., 30, 1123 (1971), Abstract.
12. Wixom, R. L., Sheng, D. Y.-B., and Becker, R. S. Utilization of organic nitrogen compounds by Hydrogenomonas eutropha. Biotechnol. Bioeng., 14: 985-1006 (1972).
13. Semeraro, R. J., and Wixom, R. L. Studies in valine biosynthesis. X. The acetohydroxyacid synthetase from Rhodopseudomonas spheroides. - MSS in preparation.

FINAL COMPOSITE PROGRESS REPORT

Project Title: Application of Nitrogen Metabolism in Autotrophic Bacteria to Chemosynthetic Bioregeneration in Space Missions.

Project Sponsor: National Aeronautics and Space Administration.

Project Number: NASA No. NGR 26-003-~~023~~, University of Missouri, No. 5814-2229.

Project Initiated: 10-1-67 (fiscal start).

Principal Investigator: Dr. Robert L. Wixom
Associate Professor of Biochemistry and
Research Associate, Space Sciences Research Center,
School of Medicine
University of Missouri
Columbia, Missouri 65201

Period covered by report: 10-1-67 to 9-30-69.

Report prepared: 10-20-69.

Scope of report: Includes data previously submitted in three Semiannual Progress Reports on 4-1-68, 10-1-68 and 4-1-69 and the past six month's research.

Abstract

The chemolithotroph, Hydrogenomonas eutropha, is being considered as a life support, bioregenerative system. This project focuses on several metabolic functions that are related to the proposed nitrogen cycle between man and this microbe. Specifically this organism has the capability to utilize as the sole nitrogen source such urine components as urea and fifteen individual amino acids, but not nine other amino acids. The effectiveness of utilization was high for many amino acids. Several specific growth inhibitions were also observed.

The enzyme that catalyzes the incorporation of ammonia in the medium into amino acids has now been identified as a NADP-specific, L-glutamate dehydrogenase. This enzyme has a constitutive nature.

This organism can ⁿsynthesize all of its amino acids from carbon dioxide and ammonia. Several of the enzymes that have been rigorously proven to participate in valine-isoleucine biosynthesis in the heterotroph, Escherichia coli, have now been found in cell-free extracts of H. eutropha, H. facilis and H. ruhlandii. Therefore with the background literature of multiple pathways of individual amino acid biosyntheses, our evidence to date is consistent with the Hydrogenomonas group having the same pathway of valine-isoleucine formation as the classical E. coli.

IA. Project Objectives

Since a key problem in prolonged human space flight is a continued source of food for the astronaut, recent NASA research has focused on investigation of unicellular organisms as a food-manufacturing, waste-conversion unit in a self-sustaining, life-support system. While others have examined the photosynthetic algal system, this project has centered on autotrophic bacteria and particularly on the utilization of nitrogen and amino acid-protein metabolism in these microbes. Hydrogenomonas would derive its energy for cellular synthesis by the oxidation of hydrogen from water electrolysis. It would use the nitrogen in urine and, perhaps also, feces for the biosynthesis of its amino acids and then its cell protein. After appropriate processing, these microbial cells would be utilized as a source of protein and other nutrients for human food. These metabolic steps would be necessary to complete and to balance the carbon, nitrogen, oxygen and hydrogen in the proposed closed ecological system.

This particular project focuses on the microbial part of the nitrogen cycle, namely the utilization of nitrogenous compounds found in urine and feces, the biosynthesis of the microbial amino acids, and amino acid-protein metabolism in these microbes. As stated in the original NASA grant application, the emphasis of these investigations continues to be on microbial growth, the enzymes related to these transformations, and their metabolic adaptation or control.

IB. Summary of Two Year's Activities in Project

Introduction - In this Final Progress Report, the experimental results in Section IBa are described in considerable detail with a portion of the data in Tables or Figures. Since Section IBb has been accepted for publication in *Biochim. Biophys. Acta*, the description here is very limited. The data in Section IBc and IBd is described in abstract form, since preparation of a full journal manuscript is under way.

The earlier, complete, 17-year literature search on Hydrogenomonas in Chemical Abstracts, Biological Abstracts, and STAR has 153 references, which are grouped by subject content. This bibliography has been invaluable as a guide to the past literature, for design of present experiments and deducing future research directions. This search has provided knowledge of a closed ecological system for prolonged human space travel - i.e. a balanced symbiotic relationship between man and a biological factory as a source of man's essential amino acids, carbohydrate, and lipids. Since other investigators have examined in some detail the oxidation of hydrogen, electron transport, CO₂ fixation, polymer formation, gas ratios, pilot plant projects, and various bioengineering questions on Hydrogenomonas sp., our emphasis has been on its nitrogen metabolism. At the time of grant application, our area of research had not been studied by earlier investigators and represented fundamental studies on the proposed symbiotic nitrogen cycle between man and Hydrogenomonas.

Some of the experimental work was supported by the Space Sciences Research Center, University of Missouri, which is hereafter abbreviated SSRC.

Iba. Utilization of Nitrogenous Compounds by Hydrogenomonas as Related to Life Support Systems

Project Report - The ability of Hydrogenomonas to grow on the nitrogenous substances found in human urine has been explored by testing these pure compounds singly and in pairs. To compensate for some biological variability, the following observations are derived from duplicate experiments with triplicate, 25 ml Erlenmeyer flasks. These flasks were incubated in stainless steel, anaerobic jars with a $H_2:O_2:CO_2$ (7:2:1) gas phase and equilibrated by rotary agitation in a 30° water bath. The conditions of gas mixing, salts medium, and 30° incubation with rotary agitation followed those of Repaske (J. Bacteriol. 85, 418-422 (1962)).

Under these conditions, the L-isomers of aspartic acid, alanine, glutamic acid, glutamine, and asparagine and urea were readily utilized as the sole sources of nitrogen at 24 and 48 hrs. growth (Table I). The nitrogen of proline, leucine, glycine, phenylalanine, threonine, histidine, and serine was only slowly released during the first 24 hours of growth, but gave good growth at the 48 hour reading. Tryptophan, isoleucine, and valine gave intermediate results. H. eutropha failed to grow significantly within 48 hours when hydroxyproline, arginine, lysine or methionine was the sole nitrogen source in the medium (Table I). These results are similar to those briefly reported recently by Fraser-Smith, E. C. B., Austin, M. A. and Reed, L. L. (J. Bacteriol. 97, 457-459 (1969)). De Cicco, B. T. and Stukus, P. E., reported last year that H. eutropha utilized histidine, tryptophan and alanine (J. Bacteriol. 95, 1469-1475 (1968)). Blake, E. E. in a 1966 M.S. thesis reported the utilization of L-glutamate, L-alanine and L-tyrosine (STAR, 21, N66-10956 (1966), abstract).

Since the methyl histidines are found in human urine in significant amounts, they were tested under the above conditions. L-1-Methyl histidine and L-3-methyl histidine did not support growth, unlike L-histidine which gave intermediate growth. Since arginine did not support growth, other members of the Krebs-Henseleit urea cycle were tested. L-Ornithine and L-citrulline were also found to be non-utilizable for growth. Taurine is found in human urine, and was found to support growth under our conditions.

Variation of the NH_4Cl concentration in the medium confirmed the finding that 0.019 M was the optimal concentration (Fig. 1). Seven nitrogenous compounds were selected to test for growth, each at 0.001, 0.002, 0.003, 0.005, 0.010 and the usual 0.019 M final nitrogen concentration in the medium (Fig. 2). The growth response curves at 48 hours were approximately the same for NH_4Cl , L-alanine, L-glutamate, L-glutamine, and urea; the curves for glycine and histidine were slightly lower. The growth at the 0.001 M nitrogen concentration was 17 to 32% that of the 0.019 M NH_4Cl control.

The next objective was to search for the possible interactions of the utilizable amino acids and the slowly metabolized amino acids. These interactions might be: inhibition of growth of the utilizable amino acid, facilitation of growth of the so-called slowly metabolized amino acid and other more complex responses. The nature of these interactions should be defined in view of the proposed use of H. eutropha in a closed cycle, bioregenerative system. More specifically, since the poorly utilized amino acids will accumulate in a growth media based on human urine over prolonged growth periods, the following series of experiments was performed (Table II). The eight readily utilized nitrogen sources were added to the Erlenmeyer flasks at the suboptimal concentration of 0.001 M of nitrogen in the test compound and the seven non-utilized amino acids at the usual concentration of 0.019 M. The growth data indicates that tryptophan or arginine, in combination with one of the utilizable nitrogen sources, always promoted growth to a greater extent than either alone and, in some cases, to a greater extent than the sum of each pair of individual figures. Presumably the catabolism of tryptophan or arginine in a mixture in the medium was greater under these conditions than when present from the initiation of growth as the sole nitrogenous source. Isoleucine or valine with the same combinations promoted growth to a greater extent than either alone, with the exception of combination with L-glutamate or L-glutamine respectively. Hydroxyproline and methionine, in addition to being metabolically inert, inhibited the microbe's utilization of nitrogen from almost all sources tested, except histidine and glutamine. Other more individualized results of both growth promotion or inhibition were noted for the other combinations of amino acids (Table II).

H. eutropha was also autotrophically grown in 1-1 Erlenmeyer flasks with rotary agitation at 30° with a reproducible generation time of about 2.0 - 2.5 hours for NH_4Cl , urea, or alanine; the maximum turbidity was about the same for these tubes. Use of a 30° water - jacketed, tissue culture, spinner flask with the fastest possible magnetic stirring gave poor growth (7.0 hr generation time). At Fe concentration of 8 mg/l, FeSO_4 , $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, FeCl_3 or $\text{Fe}(\text{NH}_4)\text{SO}_4)_2$ led to the same generation time and same maximum turbidity. Consequently subsequent experiments have continued to use Repaske's FeSO_4 at the above concentration.

To utilize these findings on a larger scale with multiple simultaneous comparisons and to harvest sufficient cells for enzyme experiments, a new incubation system was devised with 6 high speed magnetic stirrers underneath a plexiglass 30° water bath containing flasks linked to the gas distribution system. The new stirrers (max. speed - 1700 RPM) led to entrainment of gas bubbles in the liquid medium. The optimal conditions to achieve a reproducible time of 1.8 - 2.2 hours were use of a 1.5 inch magnetic bar rotating at about 1300 RPM and absence of the baffles in the flasks. These conditions were maintained for the following series of experiments for variation of gas phase, nitrogen source, and carbon source. Whenever air was substituted for the $\text{H}_2:\text{O}_2:\text{CO}_2$ gas mixture for growth on alanine, glucose plus NH_4Cl , or glucose plus alanine, the generation time was prolonged and turbidity readings at 48 hours were lowered. Alanine as the sole nitrogen source with the 1.0 M CO

gas mixture for growth on alanine, glucose plus NH_4Cl , or glucose plus alanine, the generation time was prolonged and turbidity readings at 48 hours were lowered. Alanine as the sole nitrogen source with the $\text{H}_2:\text{O}_2:\text{CO}_2$ gas phase gave the same generation time as NH_4Cl , but lower maximum growth. Growth on alanine in air with its lowered CO_2 supply impaired growth by both criteria; but since positive growth occurred in these flasks, H. eutropha can degrade the carbon skeleton of alanine and reutilize the carbon for synthesis of other cellular components. Heterotrophic growth, achieved by addition of glucose, gave a similar growth response to chemolithotrophic growth with NH_4Cl or alanine as the nitrogen source. These studies are also related to the efficient reutilization of urinary nitrogen through bacterial protein to complete the nitrogen cycle.

These results with exact concentrations and identities of amino acids can be reviewed in the light of information on the known composition of urinary nitrogen compounds. The α -amino nitrogen concentration in normal human adult urine is 0.3-0.7 gm N per day. Within this nitrogen fraction are found the following amino acids (arranged in decreasing order of concentration): glycine, 71-416 $\text{mM} \times 10^2$ per 24 hours; taurine, 22-185; histidine, 13-137; 1-methyl-histidine, 13-93; glutamine and asparagine, 29-77; serine, 21-62; 3-methyl-histidine 18-52; alanine, 6-50, and variable lower concentrations of threonine, tyrosine, phenylalanine, isoleucine, leucine, cystine; lysine, glutamic acid, valine, aspartic acid, methionine, arginine and ornithine. Comparison of our H. eutropha growth data and this urinary composition indicates that 1- and 3- methyl-histidines, methionine, lysine and arginine were not utilized by H. eutropha (Table I). Lysine and methionine were poorly utilized in a mixed nitrogen source, and may be predicted to accumulate during continuous growth of H. eutropha on urine in a bioregenerative system.

In addition to these amino acids, other nitrogenous compounds are found in urine. A variety of project reports to NASA describe Hydrogenomonas strains that have been found to utilize urea, the main nitrogenous component of urine (see next Section IBb). Autotrophically-grown H. eutropha can utilize the nitrogen of uric acid, allantoin, hippuric acid and urea, but not creatinine (Ammann, E. C. B., and Reed, L. J., Biochim. Biophys. Acta 141, 135-143 (1967). H. facilis utilizes thymine, cytosine, and uracil, whereas Hydrogenomonas H16 degraded only cytosine (Kaltwasser, H. and Kramer, J., Arch. Mikrobiol. 60, 172-181 (1968). All of the above studies and our investigations are related to the problem of efficient reutilization of urinary nitrogen through bacterial protein, human dietary protein, and repetition of the cycle.

Personnel on Project - Mr. R. S. Becker, Mr. K. S. Suh and Dr. R. L. Wixon.

Degree of NASA Support - Joint with SSRC research grant.

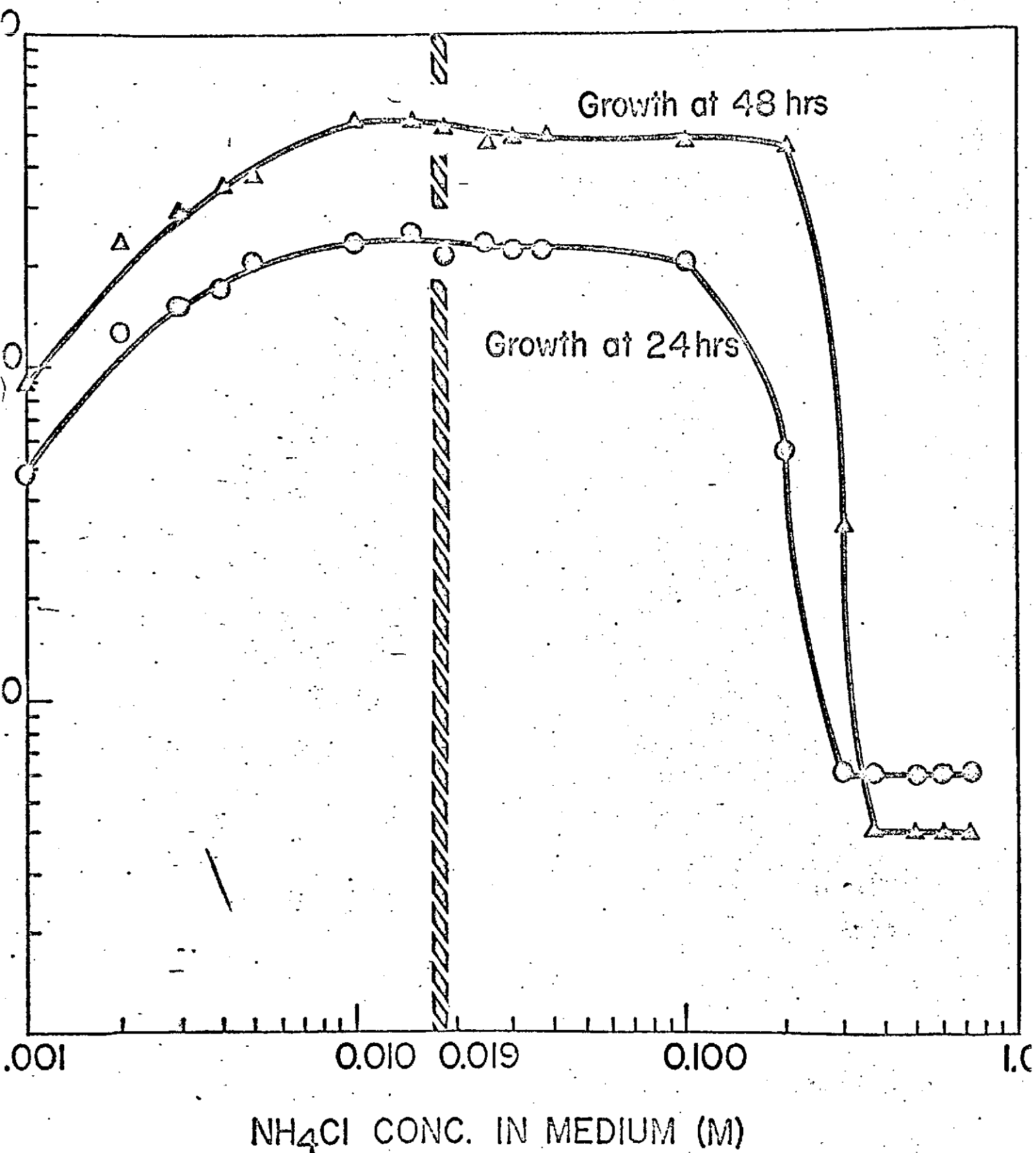
Pertinent Publications Completed or in Preparation - See II 4 and 8.



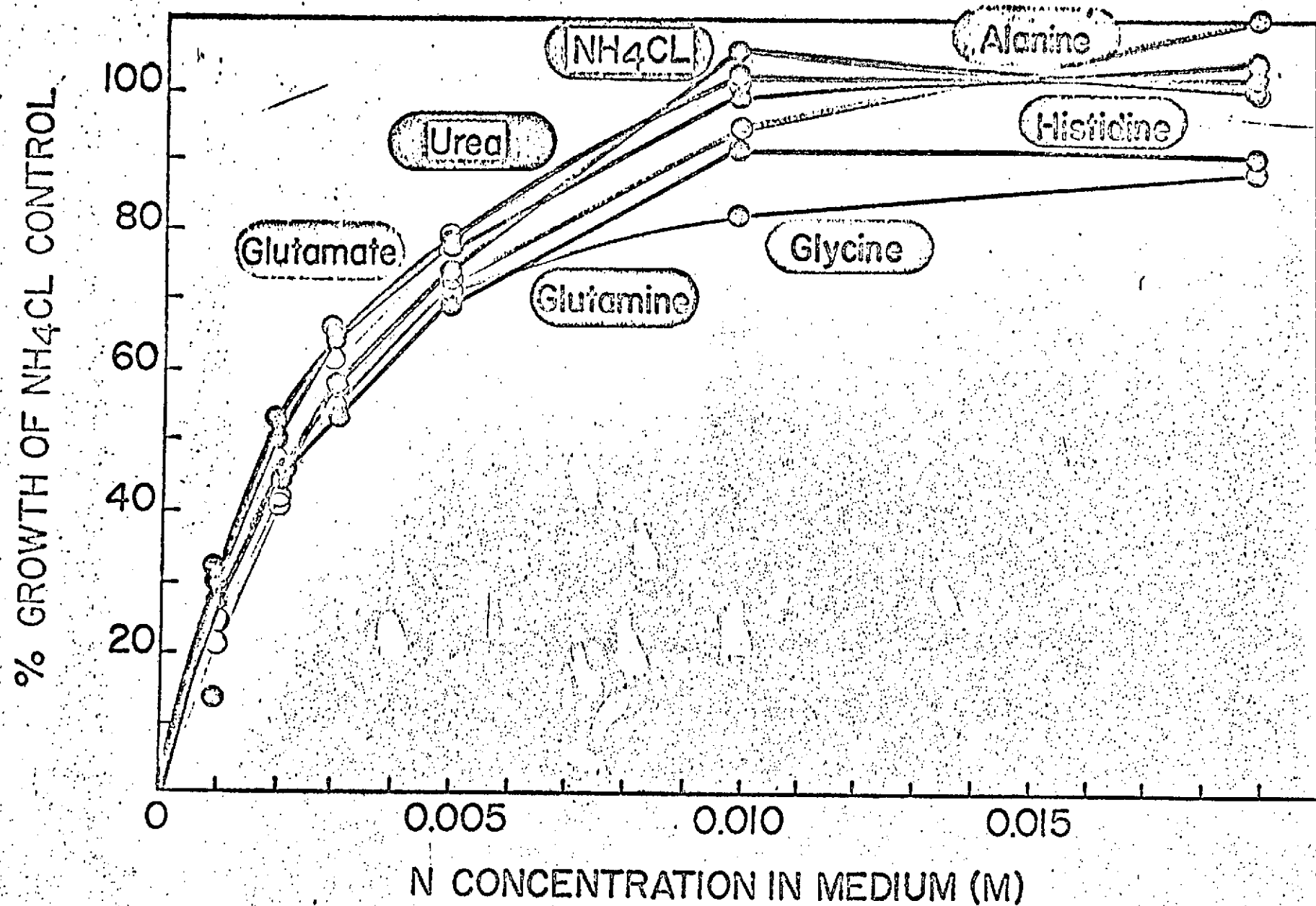
GROWTH OF *H. EUTROPHA* WITH SINGLE NITROGEN SOURCES

Growth characteristic	Sole nitrogen source	Av. % growth of NH ₄ Cl control	
		At 24 hrs.	At 48 hrs.
High group	Aspartate	130	97
	Alanine	126	106
	Glutamate	118	102
	Glutamine	112	102
	Asparagine	110	81
	NH ₄ Cl	100 (278*)	100 (547*)
	Urea	33	91
Medium group	Proline	33	90
	Leucine	30	97
	Glycine	30	88
	Phenylalanine	27	91
	Threonine	22	99
	Histidine	17	73
	Serine	13	86
Slow group	Tryptophan	17	40
	Isoleucine	9	37
	Valine	3	20
No growth group	Hydroxyproline	5	3
	Arginine	4	4
	Lysine	4	4
	Methionine	3	2

GROWTH OF H. EUTROPHA ON NH_4Cl



UTILIZATION OF SINGLE N SOURCES BY H. EUTROPHA



INTERACTION OF TEST AMINO ACIDS WITH UTILIZABLE NITROGEN SOURCES BY H. EUTROPHA

Test L-amino acid (<u>0.019 M</u>)	Av. % growth of 0.019 M NH ₄ Cl control							
	<u>None</u>	<u>Try</u>	<u>Arg</u>	<u>Ile</u>	<u>Val</u>	<u>Lys</u>	<u>Hyp</u>	<u>Met</u>
Utilizable N source (<u>0.001 M N</u>)								
None	—	40	4	37	20	4	3	2
NH ₄ Cl	26	46	35	72	69	35	21	23
L-Alanine	22	45	38	73	68	11	12	15
L-Glutamate	23	59	25	25	40	14	19	11
L-Glutamine	26	51	31	51	22	48	29	20
Urea	37	104	53	108	74	6	13	35
Glycine	27	65	47	54	65	25	23	5
L-Histidine	26	80	35	42	45	32	25	32

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Table II

IBb. Enzymes in Nitrogen Metabolism in *H. eutropha*

Project Report - A review of the literature (our own continuing bibliography and that by Dr. J. Pulliam and F. Hong, G. Washington University Medical Center, Washington, D. C., NASA contract no. NSR-09-010-027, September, 1968) indicated that the enzymatic route of ammonia assimilation in Hydrogenomonas has not been demonstrated to date. Earlier investigators had shown that Hydrogenomonas extracts contained glutamate dehydrogenase activity, but in view of two other known microbial routes of ammonia assimilation, the mere presence of an enzyme does not prove the existence of a pathway. Since this area of our research has been written and accepted for publication, a photocopy of this manuscript for Biochim. Biophys. Acta is attached in lieu of description here.

For understanding metabolism, it is not sufficient to know solely that certain enzymes are present in a microbe; such knowledge must be coupled with both the biosynthesis and function of the enzyme under various conditions of medium, gas phase, growth cycle, continuous culture, and other environmental factors. To use more modern phraseology, the next research phase would be to elucidate the metabolic control mechanism(s) for the pertinent enzymes. Therefore, pending continued adequate research support, future research should emphasize the search for possible controls such as induction, repression, end product inhibition, allosteric effectors, subunit structure and other even more refined strategies for regulation of cellular events.

Our initial NASA grant application (prepared 9-1-66) raised a variety of questions and proposed experiments on the enzymatic utilization of urea. Shortly thereafter, the responsible enzyme in Hydrogenomonas H16 was identified as urease (König, C., Kaltwasser, H., and Schlegel, H. G., Arch. Mikrobiol. 53, 231-241 (1966)). Urease, found in autotrophically and heterotrophically grown cells, was induced by urea and repressed by ammonium salts. Urease underwent an oscillatory change during the growth cycle of Hydrogenomonas H16 (König, C., and Schlegel, H. G., Biochim. Biophys. Acta 139, 182-185 (1967)). With awareness of these recent, careful studies, we have pursued other aspects in our original NASA grant proposal.

The enzyme responsible for utilization of uric acid, another urinary nitrogenous component, by Hydrogenomonas H16 has been demonstrated to be uricase (Kaltwasser, H., Arch. Mikrobiol. 60, 160-171 (1968)). Uricase was found to be an inducible enzyme and found in a particulate fraction.

To return to our investigations, we have recently found glutaminase- and asparaginase activities in cell-free extracts of H. eutropha. Continued exploration of this finding and other enzymes in nitrogen metabolism will depend on an adequate source of funds.

Personnel on Project - Mr. A. A. Joseph, Mr. R. S. Becker and Dr. R. L. Wixom.

Degree of NASA Support - 100%.

Pertinent Publications Completed or in Preparation - See II-4, 7, and 8.

IBc. Valine-Isoleucine Biosynthesis in Hydrogenomonas

Introduction - The pathways of the biosynthesis of amino acids in microorganisms have been deciphered in considerable detail for Escherichia coli and Neurospora crassa by mutant studies, radioisotope tracer experiments, and enzymatic investigations. Then other diverse microorganisms and plants were examined. After the initial reporting period of apparently identical pathways, it became apparent that there are two different pathways for the formation of lysine, glutamic acid and tryptophan in different microbial systems. The metabolic regulation of these biosynthetic pathways is even more heterogeneous. For instance, five different patterns of regulation of the formation of the aspartate family of amino acids are now known. The possibility of diversity in the biosynthetic routes in the chemolithotrophic bacterium has not^{been} previously studied.

The pathway of valine-isoleucine formation involves four parallel biosynthetic steps by common enzymes, namely acetolactate synthetase, reductoisomerase, dihydroxyacid dehydratase, and an aminotransferase. The evidence for this pathway in E. coli and N. crassa has accumulated from the research of many investigators. Although the seven previous publications by this author have demonstrated this pathway in many other heterotrophs and many higher plants, there has been to date essentially no investigations other than the authors on amino acid biosynthesis in the chemolithotrophs.

Project Report - Research on the chemolithotrophic Hydrogenomonas proceeded by searching for the biosynthetic enzyme, dihydroxyacid dehydratase, E. C. 4.2.1.9. This enzyme is in the middle of the E. coli valine-isoleucine biosynthesis, and has a unique substrate. Log phase cells of heterotrophically grown H. facilis, heterotrophic and autotrophic, H. ruflandii and H. eutropha were harvested, ruptured by ultrasonic oscillation and assayed for dihydroxyacid dehydratase. These extracts catalyzed the dehydration of DL- α , β -dihydroxyisovaleric acid and where tested, DL- α , β -dihydroxy- β -methyl-n-valeric acid to their corresponding keto acids. These compounds are known intermediates of respectively valine and isoleucine biosynthesis in Escherichia coli. The optimal pH of the dehydratase from autotrophic H. eutropha was pH 8.0-8.2. The enzymatic product was identified as α -ketoisovalerate by thin layer chromatography of the 2,4-dinitrophenylhydrazine derivatives on silica gel as compared with other known ketoacid hydrazones. This extract catalyzed the dehydration of DL- α , β -threo-dihydroxybutyrate at a faster rate than the erythro isomer, but did not alter 3 other dihydroxy acids. Serine dehydratase activity was found, but the dehydratases or desulphhydrases for threonine, homoserine, cysteine, and homocysteine were absent.

Finding the above serine dehydratase activity led to experiments to differentiate these several activities. The addition of pyridoxal phosphate to the dehydratase assay system increased the rate of keto acid formation from L-serine, D-serine, and L-tartrate, but not from dihydroxyisovalerate as a substrate. Furthermore the addition of hydrazine or semicarbazide inhibited keto acid formation from L-serine, D-serine, and L-tartrate, but not from dihydroxyisovalerate as the substrate. Therefore these experiments demonstrate the existence of a dihydroxyacid dehydratase independent of other incidental dehydratases in the extract.

The reductoisomerase enzyme has also been demonstrated in H. eutropha extracts. Omission of the substrate, acetolactate, caused a loss of activity. Deletion of NADPH, or substitution of NADPH by NADH, led to a loss of activity. Omission of Mg^{++} did not lead to appreciable decrease of activity in this crude system.

This enzymatic evidence is consistent with the presence of the E. coli route of valine-isoleucine biosynthesis in the chemolithotrophic bacteria Hydrogenomonas eutropha.

Personnel on Project - Mr. A. A. Joseph and Dr. R. L. Wixom.

Degree of NASA Support - Shared with SSRC research grant.

Pertinent Publications Completed or in Preparation - See II - 1, 3, 4 and 9.

IBd. Metabolic Controls of Valine-Isoleucine Biosynthesis

Project Report - With the main emphasis on the previously described research areas, only shorter attention was given to this sub-research area, which was also described in the original research grant application. To introduce this subject, Cutinelli, et al. (Arkiv. Kemi 3, 315-320 (1951) found that C^{14} -acetate was incorporated into valine and other amino acids of the photoorganotroph, Rhodospirillum rubrum, in a different pattern from that previously obtained in Escherichia coli and Neurospora crassa. Thus, search for the presence of the valine-isoleucine biosynthetic enzymes in R. rubrum and Rhodopseudomonas spheroides, two members of the non-sulfur purple bacteria of the Athiorhodaceae family was undertaken. Cell-free extracts of R. spheroides and R. rubrum, grown aerobically in the dark and anaerobically in the light, were found to catalyze the conversion of α, β -dihydroxyisovaleric acid to a keto acid. Dehydratase activity was present when cells were grown with glutamate-malate or acetate as carbon sources. Other characteristics of this dihydroxyacid dehydratase were described earlier (First NASA Semiannual Progress Report; Ref. 1 and 2). The activities of a valine-glutamate aminotransferase, reductoisomerase and acetohydroxyacid synthetase were also demonstrated several years ago and set the stage for the following new studies of one of several possible metabolic controls in the photosynthetic bacteria.

The first enzyme in the valine pathway is acetohydroxyacid synthetase; it was first studied in R. spheroides extracts in the summer of 1968 during the tenure of a medical student research fellowship. The required

components in its assay system were shown to be phosphate buffer, pH 7.4, pyruvate, thiamin pyrophosphate, Mg^{++} , FAD, and extract. The product of the reaction was α -acetolactate, a known intermediate in the E. coli pathway of valine biosynthesis. After an initial lag, the enzyme activity was proportional to the time of reaction. Activity was also proportional to low pyruvate concentration; high concentrations caused substrate inhibition. The following characteristics were determined: optimal pH - 7.4, K_m pyruvate - $4.5 \times 10^{-3}M$, K_m TPP - $2.96 \times 10^{-5}M$, and K_m Mg^{++} - $1.01 \times 10^{-3}M$. Addition of FAD at 5 μM enhanced the activity by 2.5 fold. The enzyme was sensitive to endproduct inhibition by valine in a competitive pattern according to a double reciprocal plot. Since isoleucine, leucine, alanine, phenylalanine, lysine, and glutamic acid were not inhibitory, the inhibition was specific for valine.

To summarize, the results of these experiments demonstrate that the E. coli pathway of valine biogenesis also occurs in the above photosynthetic bacteria, and that endproduct inhibition is at least one of the mechanisms to regulate valine biosynthesis in this photosynthetic bacterium.

Personnel on Project - Mr. R. J. Semeraro and Dr. R. L. Wixom.

Degree of NASA Support - Small amount of funds for consumable supplies - shared with SSRC research grant.

Pertinent Publications Completed or in Preparation - See II - 6, 9 and 10.

II. List of Recent Publications Related to Grant

1. Wixom, R. L., Joseph, A. A., Hwang, S. W., and Semeraro, R. J., Comparative Aspects of Valine-Isoleucine Biosynthesis. Seventh International Congress of Biochemistry, Tokyo, Japan, August 19-25, 1967; Abstract; reprints forwarded earlier to NASA and served as background for Hydrogenomonas research.
2. Semeraro, R. J., and Wixom, R. L., The Pathway of Valine-Isoleucine Biosynthesis in Photosynthetic Bacteria. Trans. Mo. Acad. Sci. 1, 82 (1967); Abstract; reprints forwarded earlier to NASA and served as background for Hydrogenomonas research.
3. Joseph, A. A., and Wixom, R. L., The Biosynthesis of Valine and Isoleucine in Autotrophic Bacteria. Society for Experimental Biology and Medicine, Annual Meeting of the Missouri Section at the University of Missouri, Columbia, Mo., December 9, 1967; Abstract; reprints forwarded earlier to NASA.
4. Joseph, A. A., Amino Acid Metabolism in Propionibacteria and Hydrogenomonas. Thesis for Ph.D. degree, University of Missouri, December 19, 1968.
5. Wixom, R. L., and Joseph, A. A., Ammonia Assimilation in Hydrogenomonas. Eleventh West Central States Biochemistry Conference, November 2, 1968, Ames, Iowa; Abstract.
6. Wixom, R. L., and Semeraro, R. J., Valine Biosynthesis in Rhodopseudomonas spheroides. Federation Proc., 28, 910 (1969); Abstract; reprints enclosed.
7. Joseph, A. A., and Wixom, R. L., Ammonia Incorporation in Hydrogenomonas, Biochim. Biophys. Acta, manuscript submitted for publication July 29, 1969 and accepted for publication September 5, 1969; a complete photocopy is attached.
8. Wixom, R. L., Joseph, A. A., and Becker, R. S., Amino Acid Metabolism in Hydrogenomonas eutropha. 158th National Meeting of the American Chemical Society, New York, N. Y., September 9-12, 1969; Abstract; Xerox copies enclosed.
9. Wixom, R. L., Heineman, M., and Semeraro, R. J., Studies in valine biosynthesis IX. The enzymes in photosynthetic and autotrophic bacteria; manuscript in preparation.
10. Semeraro, Russell J., Valine-Isoleucine Biosynthesis in Photosynthetic Bacteria, Thesis in preparation for Ph.D. degree, University of Missouri.

III. Research Personnel Associated with NASA-SSRC grants

Dr. Robert L. Wixom, Principal Investigator of NASA grant, Associate Professor of Biochemistry, Research Associate in Space Sciences Research Center, University of Missouri, Columbia, Mo.

Dr. Augustine A. Joseph, Ph.D. degree completed December 19, 1968; thesis entitled, Amino Acid Metabolism of Propionibacteria and Hydrogenomonas; half-time Research Assistant on NASA grant as graduate student from 10-1-67 to 12-30-69 on subject areas IBb and IBc; present position, Postdoctoral Research Fellow, Rockefeller University, New York, N. Y.

Mr. Russell J. Semeraro, Ph.D. candidate, degree expected February 1970; thesis entitled, Valine-Isoleucine Biosynthesis in Photosynthetic Bacteria; half-time Research Assistant on earlier SSRC project; a medical student since the fall of 1967; during the tenure of a summer 1968 medical student research fellowship, he completed his final thesis experiments, for which only small amounts of SSRC and NASA Consumable Supply funds were used for subject area IBd.

Mr. Richard S. Becker, Research Assistant on NASA grant from 2-1-68 to 8-31-69 on subject area IBa and IBb, biochemistry - microbiology background.

Mr. Ki Surk Suh, new biochemistry graduate student, U. S. citizen; half-time Research Assistant on SSRC grant in 1968 on subject area IBa.